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ORIGINAL ARTICLE

A comparative proteomics analysis in roots of soybean to compatible symbiotic bacteria under flooding stress

Amana Khatoon · Shafiq Rehman · Afshin Salavati · Setsuko Komatsu

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Abstract A proteomics approach was used to evaluate the effects of flooding stress on early symbiotic interaction between soybean roots and soil bacteria, *Bradyrhizobium japonicum*. Three-day-old soybean was inoculated with *B. japonicum* followed by flooding. The number of root hairs in seedlings, without or with flooding stress, was increased after 3 days of inoculation. Proteins were extracted from roots and separated by two-dimensional polyacrylamide gel electrophoresis. Out of 219 protein spots, 14 and 8 proteins were increased and decreased, respectively, by inoculation under flooding compared with without flooding. These proteins were compared in untreated and flooded seedlings. Increased level of 6 proteins in flooded seedlings compared with untreated seedlings was suppressed by inoculation in seedlings under flooding. They were related to disease/defense, protein synthesis, energy, and metabolism. Differential abundance of glucan endo-1,3-beta-glucosidase, phosphoglycerate kinase, and triosephosphate isomerase, based on their localization in middle and tip of root, indicated that they might be related to increase in number of root hairs. These results suggest that disease/defense, energy, and metabolism-related proteins may be particularly subjected to regulation in flooded soybean seedlings, when inoculated with *B. japonicum* and that this regulation may lead to increase in number of root hair during early symbiotic differentiation.

Keywords Soybean · Flooding · Proteomics · Root · Symbiosis

Abbreviations

2-DE	Two-dimensional polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
MS	Mass spectrometry
LC	Liquid chromatography
p*I*	Isoelectric point
IEF	Isoelectric focusing
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight

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A. Khatoon · A. Salavati · S. Komatsu (✉)
National Institute of Crop Science, National Agriculture and Food Research Organization, 2-1-18 Kannondai, Tsukuba 305-8518, Japan
e-mail: skomatsu@affrc.go.jp

A. Khatoon · S. Rehman
Department of Plant Sciences, Kohat University of Science and Technology, Kohat 26000, Pakistan

A. Salavati
Department of Plant Breeding and Biotechnology, Ramin University of Agriculture and Natural Resources (Khuzestan), Ahwaz, Iran

Introduction

Soybean is an important source of protein for human and animal nutrition, as well as a major source of vegetable oil (Graham and Vance 2003). Soybean has the ability to fix the atmospheric nitrogen by a symbiotic interaction with the soil bacteria, *Bradyrhizobium japonicum*, by forming specialized structures called nodules (Nap and Bisseling 1990). The process of symbiosis is started by the exchange of chemical signals between plant root and bacteria, more

specifically in response to flavonoids secreted by the plant. In response to these flavonoids, the bacteria synthesize a lipo-chitooligosaccharide known as a Nod factor (Lerouge et al. 1990). The perception of the Nod factor by the plant occurred specifically on root hairs, which leads to the deformation of root hair cells and to the activation of root cortical cell divisions leading to the formation of the nodule primordium (Libault et al. 2010).

Proteome studies of nodule-forming process have highlighted the involvement of nodulins during different stages of nodule formation including infection, nodule maturation, and maintenance to support the nitrogen fixation process (Sanchez et al. 1991). These nodulins were found to be specific to the stage of nodule formation. Early nodulin genes were detected during the infection and nodule formation stage while expression of late nodulin genes was observed at about the onset of nitrogen fixation (Nap and Bisseling 1990). Salavati et al. (2012) identified metabolism and energy production-related proteins in normal-nodulating soybean variety involved in early response to *B. japonicum* inoculation. Tesfaye et al. (2006) reported that genes for asparagine synthetase, glutamine synthetase, glycolytic enzymes malate dehydrogenase, and phosphoenolpyruvate carboxylase were up-regulated in nodules.

Soybean is sensitive to flooding which caused a decrease in photosynthesis (Chen et al. 1992), nutrient uptake (Sallam and Scott 1987), and nitrogen fixation (Sung 1993). Studies have reported that flooding stress on soybean affected the proteins involved in the process of fermentation (Russell et al. 1990), glycolysis, protein storage, and disease/defense (Hashiguchi et al. 2009). Soybean response to flooding involved up-regulation of genes involved in ethylene biosynthesis, alcohol fermentation, and cell wall loosening that might lead to an anaerobic response (Komatsu et al. 2009a). Proteomics analysis of soybean plasma membrane revealed that signaling-related proteins might be involved in the regulation of ion homeostasis under flooding stress (Komatsu et al. 2009b). Nanjo et al. (2010) clarified that flooding stress induced an imbalance of protein level involved in metabolic pathways including carbohydrate metabolism. Protein profile in soybean revealed that proteins related to cytoskeleton reorganization, cell expansion, and programmed cell death were increased, whereas ion transport-related proteins were decreased under flooding stress (Salavati et al. 2012). Furthermore, it has been investigated whether flooding caused a decrease in nitrogenase activity (Sanchez et al. 2011) and changed the protein abundance in a similar manner to nitrogen substitution (Komatsu et al. 2010).

Flooding injuries lead to reduced growth and final yield of plants (Sung 1993). Minchin and Summerfield (1976) reported that although stage of early symbiotic differentiation is sensitive to flooding stress that causes decreased number of nodules and nitrogen fixation efficiency of nodules, however, this interaction induced the ability to cope with flooding stress in better way than plants without symbiotic relationship with bacteria. Understanding of the mechanism of plant response during early stage of infection with compatible bacteria under flooding stress is therefore needed. In the present research, proteomics approach has been used to evaluate the effects of flooding stress on early symbiotic interaction between soybean roots and *B. japonicum*. Root proteins from soybean seedlings inoculated with *B. japonicum* under flooding stress were analyzed using two-dimensional polyacrylamide gel electrophoresis (2-DE) based proteomics in combination with mass spectrometry (MS). Abundance of candidate proteins was analyzed based on their localization in middle and tip of soybean roots.

Materials and methods

Bacteria culture

Bacterial strain, *B. japonicum* MAFF 211342 was obtained from the Genebank at National Institute of Agrobiological Sciences (Tsukuba, Japan). Bacterial culture was grown for 3 days at 30 °C in yeast mannitol broth medium containing 3.67 mM K_2HPO_4 (pH 6.8), 0.81 mM $MgSO_4$, 1.71 mM NaCl, 50 mM mannitol, 40 mM $CaCl_2$, and 0.04 % yeast extract. Before inoculation, *B. japonicum* culture was centrifuged for 10 min with 2,000×*g*. The pellet was washed and diluted with sterile water to a final optical density $(OD)_{600} = 0.1$.

Plant growth conditions

Soybean (*Glycine max* L. cultivar Enrei) seeds were sterilized with sodium hypochlorite solution, rinsed in water, and were germinated in sand under white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h light period/day) in a growth chamber (Sanyo, Tokyo, Japan) maintained at 25 °C and 70 % relative humidity. Three-day-old soybean was treated with *B. japonicum* inoculation, *B. japonicum* inoculation followed by flooding, and flooding. Untreated soybean served as control. Inoculation was carried out by 1 mL of *B. japonicum* per seedling and flooding condition was maintained at 2 cm of water above the sand surface. Physiological parameters including number of secondary roots, number of root hairs per 200 μm^2 of root, lengths and fresh weights of root, hypocotyl, and epicotyls were measured 3 and 6 days after *B. japonicum* inoculation, *B. japonicum* inoculation followed by flooding, and flooding. For morphology of root hairs, roots were stained with

methylene blue, observed under light microscope (4X, 10X, 20X), and the number of root hairs per 200 μm^2 of root was determined. For protein abundance analysis, roots were collected 3 days after treatment and proteins were extracted from middle portion of roots. All experiments were biologically repeated 3 times with 30 and 16 seeds per replication for physiological and protein abundance analysis experiments, respectively. The work flow of whole experiment is described in Supplementary Fig. 1. In order to establish a relationship between differential abundance of candidate proteins with morphological changes and increased number of root hairs during early infection with *B. japonicum,* root proteome from middle and tip (5 mm of terminal portion of root that is devoid of root hairs) of soybean roots was compared.

Protein extraction

A portion (500 mg) of fresh roots was ground to powder in liquid nitrogen with a mortar and pestle. The powder was transferred to 10 % trichloro acetic acid and 0.07 % 2-mercaptoethanol in acetone and the mixture was vortexed. The suspension was sonicated for 5 min and then incubated for 45 min at −20 °C. After incubation, the suspension was centrifuged at 9,000×*g* for 20 min at 4 °C. The supernatant was discarded and resulting pellet was washed with 0.07 % 2-mercaptoethanol in acetone twice. The resulting pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended with lysis buffer consisting of 8 M urea, 2 M thiourea, 5 % CHAPS, and 2 mM tributylphosphine by vortexing for 1 h at 25 °C. The suspension was centrifuged at 20,000×*g* for 20 min at 25 °C and Supernatant collected as protein extracts. Protein contents were determined using the Bradford method (Bradford 1976) with bovine serum albumin as the standard. The protein extracts (400 µg) were applied to 2-DE.

Two-dimensional polyacrylamide gel electrophoresis

Protein extracts (400 µg) in a final volume of 200 µL of lysis buffer containing 0.4 % Bio-Lyte pH 3/10 (Bio-Rad, Hercules, CA, USA) were directly loaded into a focusing tray. The immobilized pH gradient strips (3–10 NL, 11 cm, Bio-Rad) were rehydrated for 14 h at 50 V. Isoelectric focusing (IEF) was carried out with the Protean IEF Cell (Bio-Rad) using the following conditions: 250 V for 15 min with a linear ramp, 8,000 V for 1 h with a linear ramp, and finally 8,000 V at 35,000 V/h with a rapid ramp at 20 °C. After IEF, the strips were equilibrated with 6 M urea, 2 % SDS, 0.375 M Tris–HCl (pH 8.8), 20 % glycerol, and 130 mM dithiothreitol for 30 min. The last equilibration step was done with 6 M urea, 2 % SDS, 0.375 M Tris–HCl (pH 8.8), 20 % glycerol, and 135 mM iodoacetamide for 30 min. The equilibrated strips were placed onto 15 % SDS–polyacrylamide gels with 5 % stacking gels and sealed with 1 % agarose. Electrophoresis in the second dimension was performed at a constant current of 35 mA. The gels were stained with Coomassie brilliant blue (CBB).

Gel image analysis

2-DE images were obtained using a GS-800 calibrated densitometer scanner (Bio-Rad) and the position of individual proteins on gels was evaluated with PDQuest software (version 8) (Bio-Rad). The isoelectric point (p*I*) and molecular mass of each protein was determined using 2-DE standard marker (Bio-Rad). The amount of protein in a spot was estimated using the PDQuest software with local regression model normalization. The statistical significance of the results was evaluated using the Student's *t* test when only two means were compared or two-way ANOVA Duncan's multiple comparisons test otherwise ($P < 0.05$).

Peptide preparation for mass spectrometry analysis

To identify proteins in protein spots using MS, protein spots were excised from 2-DE gels and washed with water. Proteins in the excised gel pieces were reduced with 10 mM dithiothreitol in 100 mM NH_4HCO_3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The gel pieces were digested in 100 mM NH_4HCO_3 with 1 pM trypsin (Wako, Osaka, Japan) at 37 °C overnight. The tryptic peptides were extracted from the gel grains with 0.1 % trifluoroacetic acid in 50 % acetonitrile three times. The procedure described above was performed with DigestPro (Intavis Bioanalytical Instruments AG, Cologne, Germany). The resulting peptides solutions were desalted with C-Tip pipet tips (Nikkyo Technos, Tokyo, Japan). Desalted peptide solution was analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS or nano-liquid chromatography (LC) MS/MS.

Protein identification by MALDI-TOF MS

Peptides were mixed with α-cyano-4-hydroxycinnamic acid. For analysis using a Voyager-DE-RP MALDI-TOF MS (Perseptive Biosystems, Framingham, MA, USA), calibration was external, and data were collected in the reflector mode. The resulting peptide mass data were used to search this database using the MASCOT search engine (Matrix Science, London, UK). Soybean genome sequences were downloaded from the soybean genome database (Schmutz et al. 2010, Phytozome, version 7.0, http://www.phytozome.net/soybean)

and converted into FASTA format. Search parameters were fixed cysteine carbamidomethylation, variable methionine oxidation as modifications, peptide mass tolerance ±0.5 Da, fragments ions 1 Da, 1 missed cleavage and trypsin, specified as the proteolytic enzyme. Peptides were selected in the 500–4,000 Da mass range. For positive identification, the score result of ($-10 \times \text{Log}(P)$) had to be over the significance (>60) threshold level ($P < 0.05$). Four criteria were used to assign a positive match with a known protein as follows: (1) the deviation between the experimental and the theoretical peptide masses should be less than 50 ppm. (2) At least seven different predicted peptide masses needed to match the observed masses for an identification to be considered valid. (3) The coverage of protein sequences by the matching peptides must reach a minimum of 34 %. (4) The score indicates the probability of a true positive identification and it must be at least 60. The positives matches were BLASTP searched against the NCBI protein database (http://www.ncbi.nlm.nih.gov) for updated annotation and identification of homologous proteins.

Protein identification by nanoLC MS/MS

Peptides in 0.1 % formic acid were loaded onto a 300 μm ID × 5 mm C18 PepMap trap column using an Ultimate 3,000 nanoLC (Dionex, Germering, Germany). The peptides were eluted from the trap column, separated, and sprayed using 0.1 % formic acid in acetonitrile at a flow rate of 200 nL/min on a nano-capillary column (NTTC-360/75-3, Nikkyo Technos) with a spray voltage of 1.8 kV. A nanospray LTQ XL Orbitrap MS (Thermo Fisher Science, San Jose, CA, USA) was operated in data-dependent acquisition mode with the installed XCalibur software. Full scan mass spectra were acquired in the Orbitrap over 150–2,000 *m/z* with a resolution of 15,000. The three most intense ions above the 1,000 threshold were selected for collision-induced fragmentation in the linear ion trap at normalized collision energy of 35 % after accumulation to a target value of 1,000. Dynamic exclusion was employed within 30 s to prevent repetitive selection of peptides. Acquired MS/MS spectra were converted to individual DTA files using BioWorks software (version 3.3.1) (Thermo Fisher Science). The following parameters were set to create a list of peaks: parent ions in the mass range with no limitation, 1 grouping of MS/MS scans, and threshold at 100. The resulting peptide sequence data were used to search the soybean peptide database obtained from the soybean genome database using the MASCOT search engine. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. The search parameters were peptide mass tolerance 10 ppm, fragment mass tolerance 0.2 Da, maximum missed cleavages 1 and peptide and charges +1, +2, and +3. The minimal requirement for accepting a protein as identified was as follows: (1) the score indicates the probability of a true positive identification and it must be at least 100. (2) At least three peptide sequence matches above the identity threshold. (3) The coverage of protein sequences by the matching peptides must reach a minimum of 14 %. The positives matches were BLASTP searched against the NCBI protein database for updated annotation and identification of homologous proteins.

Functional assignment of proteins and cellular localization

Identified proteins were searched in Universal Protein Resource (http://www.uniprot.org/) and Phytozome (http://www.phytozome.net/) and were annotated to their biological function according to Bevan et al. (1998). Identified proteins were analyzed with WoLF PSORT prediction (Horton et al. 2007; http://wolfpsort.org/) to predict their subcellular localization.

Results

Growth characteristics of soybean in response to inoculation with compatible bacteria under flooding stress

Condition of plant growth was analyzed to evaluate the effects of flooding stress on early symbiotic interaction between soybean roots and *B. japonicum*. Three-day-old soybean was treated with inoculation, inoculation followed by flooding, and flooding. Untreated soybean served as control. Parameters of growth condition were lengths and fresh weights of root, shoot, number of secondary roots, and number of root hairs per 200 μm^2 of root (Fig. 1; Supplementary Figs. 2 and 3). Lengths of root and shoot were decreased 3 and 6 days after flooding (Fig. 1a). The lengths of root (Fig. 1b) and shoot were increased 3 and 6 days after inoculation, respectively, (Supplementary Fig. 2). The fresh weights of root (Fig. 1b), shoot (Supplementary Fig. 2), and number of secondary roots (Fig. 1b) were decreased 3 days after flooding stress. Number of root hairs was decreased under flooding stress; however, inoculation increased the number of root hairs in seedlings without or with flooding stress (Fig. 1b). Root hair deformation and curling was also started at 3 and 6 days after inoculation (Supplementary Fig. 3). Based on the results of number of root hairs, soybean roots were collected at 3 days after inoculation, inoculation followed by flooding, and flooding, for proteomics analysis.

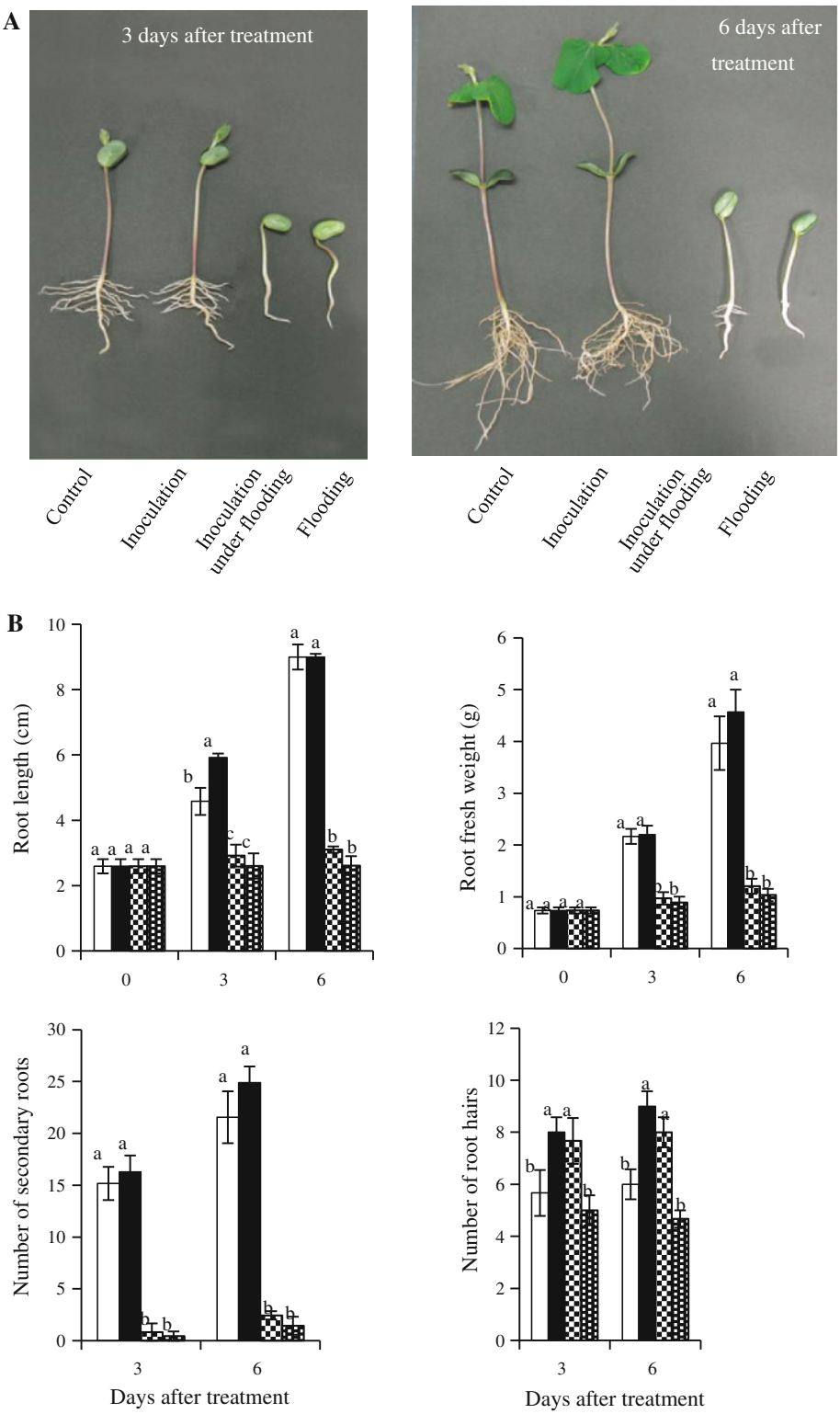

Fig. 1 Morphological comparison of growth response between untreated soybean seedlings, inoculated seedlings, seedlings inoculated under flooding, and flooded seedlings. Three-day-old soybean was inoculated with *B. japonicum* under flooding. Untreated soybean served as control. Whole seedling response of soybean was photographed 3 and 6 days after treatment (**a**). Length and fresh weight of roots, number of secondary roots, and number of root hairs were determined on 0, 3, and 6 days after treatment (**b**). Six seedlings were randomly selected for measurement at each time interval for each replication and experiment was biologically replicated three times. Each bar represents the average ±SE of 18 seedlings. *White*, *black*, *squared,* and *dotted bars* represent untreated seedlings, inoculated seedlings, inoculated seedlings under flooding, and flooded seedlings, respectively. Statistical analysis was performed by one-way ANOVA Duncan's multiple comparisons test. *Different letters* above the bars indicate a statistically significant difference ($P < 0.05$)

Differential abundance of proteins in soybean roots inoculated with compatible bacteria under flooding

A proteomics technique was used to understand the mechanism of *B. japonicum* interaction with soybean roots at early growth stage under flooding stress. Three-day-old soybean was inoculated and grown without or with flooding stress. Proteins were extracted from roots at 3 days after treatment and separated by 2-DE (Fig. 2a). The different proteome gels were compared based on molecular

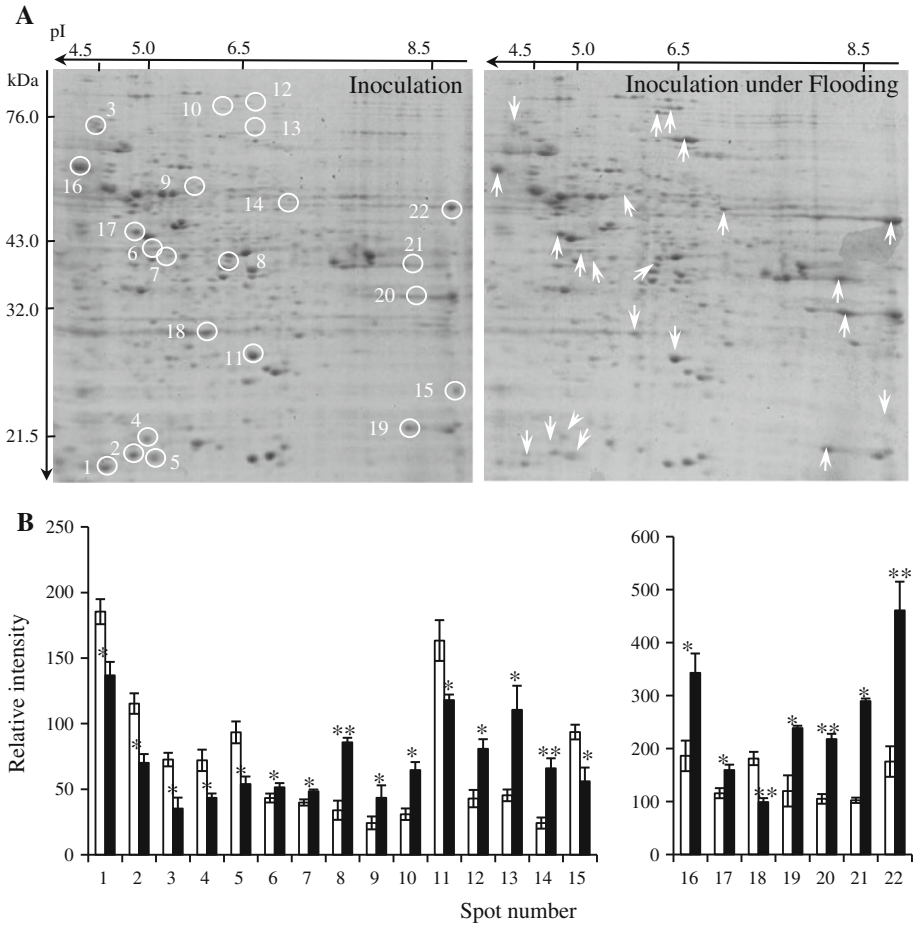

Fig. 2 2-DE pattern and level of protein abundance from soybean roots inoculated with compatible bacteria under flooding stress. Three-day-old soybean was inoculated with *B. japonicum* and flooded. Three days after treatment, proteins were extracted from roots of soybean seedlings inoculated with *B. japonicum* (*white bars*) and inoculated with *B. japonicum* under flooding stress (*black bars*), separated by 2-DE, and stained by CBB (**a**). *Open circles* show protein spots with altered abundance. *Upward* and *downward arrows* indicate increased and decreased level of proteins, respectively. The differential abundance of proteins was quantified using PDQuest software and plotted as the relative intensity (**b**). Results are presented as mean ± SE of relative protein intensity for gels from three biological replicates. The results were compared using Student's *t* test as shown by asterisks, indicating significant differences between treatments (*$P < 0.05$, **$P < 0.01$)

mass and p*I* of the protein spots. On 2-DE pattern, 219 protein spots were reproducibly detected using PDQuest software. Out of 219 protein spots in inoculated roots, 14 and 8 proteins were significantly increased and decreased, respectively, in inoculated seedlings under flooding (Fig. 2b). Of the 22 protein spots, 10 proteins had more than a 1.5-fold change in amount between the two treatments (Fig. 2b).

Identification of increased and decreased proteins in soybean roots inoculated with compatible bacteria under flooding

Differentially changed protein spots in soybean roots with *B. japonicum* inoculation under flooding stress were identified using MS (Supplemental Table 1). Protein spots from all gels were identified. A total of 25 proteins from 22 differentially changed protein spots were identified as the significant changed proteins in inoculated seedlings under flooding (Table 1). The identified proteins were classified based on their presumed biological function (Bevan et al. 1998). Energy and metabolism-related proteins were increased, whereas proteins related to disease/defense were decreased in inoculated roots under flooding (Table 1; Fig. 3a). The identified proteins were analyzed by WoLF PSORT prediction (Horton et al. 2007) to predict their subcellular localization (Fig. 3b). Sixteen proteins were localized in cytoplasm and five proteins in chloroplast. In the cytoplasm, 11 and 5 proteins were increased and decreased, respectively, whereas in chloroplast 3 proteins were decreased (Table 1; Fig. 3b).

Comparison of protein abundance level in soybean roots inoculated with compatible bacteria under flooding

In order to analyze the effect of flooding stress on protein abundance during early symbiotic differentiation, abundance of 22 proteins, which differentially changed in soybean roots inoculated with *B. japonicum* under flooding stress, was compared with untreated and flooded seedlings using 2-DE (Supplementary Figs. 4 and 5). Out of 22 protein spots, 11 proteins (spots 1, 2, 8, 9, 11, 15, 16, 18, 20, 21 and 22) were increased under flooding as compared with untreated seedlings, whereas 6 proteins (spots 6, 10, 12, 13, 14 and 17) were decreased (Fig. 4). Of the 11

Table 1 Differentially expressed proteins in soybean roots inoculated with compatible bacteria under flooding stress

Spot no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	Mr (kDa)/p*I*		FC[i]	*P* value[j]	MS[k]	Classification	
							Theo.[g]	Exp.[h]				Function[l]	Localization[m]
1	Stress induced protein SAM22	P26987	154	62	6	249	16.8/4.9	17.0/4.8	0.73	0.020	MS/MS	DisDef	Cyto
2	Glycine rich protein	ABA54143.1	186	21	3	139	19.2/6.3	18.7/5.0	0.60	0.010	MS/MS	Transcr	Nucl
3a	Chloroplast heat shock protein	ABZ04081.1	167	14	8	1,152	73.9/5.2	74.6/4.8	0.50	0.010	MS/MS	DisDef	Chlo
3b	Protein disulfide isomerase like protein precursor	NP_001238028.1	166	23	11	885	62.6/4.7	74.6/4.8	0.50	0.010	MS/MS	DisDef	Chlo
4	Superoxide dismutase (Cu–Zn)	NP_001235298.1	130	25	3	284	15.3/5.3	20.3/5.2	0.60	0.030	MS/MS	Met	Cyto
5	40S ribosomal protein	XP_003530295.1	159	60	8	254	15.2/5.5	18.3/5.1	0.57	0.010	MS/MS	ProtSyn	Cyto
6	Phosphoglycerate kinase	AAF85975.1	173	50	15	733	42.4/5.9	43.8/5.2	1.18	0.030	MS/MS	Ene	Cyto
7	Unknown	ACU19182.1	63	45	18	587	35.2/6.1	42.5/5.2	1.21	0.030	MS	Unclassi	Nucl
8	Phosphoglycerate kinase	XP_003531483.1	220	25	7	775	42.4/6.3	41.4/5.6	2.50	0.030	MS/MS	Ene	Chlo
9	UDP-glucose 6-dehydrogenase	NP_001238410.1	68	34	13	931	53.6/5.7	56.2/5.4	1.78	0.010	MS	Met	Cyto
10	Lipoxygenase	AAA03728.1	5,136	55	51	1,723	96.9/5.8	78.8/5.5	2.08	0.010	MS/MS	Met	Cyto
11	Triosephosphate isomerase	ABA86966.1	628	73	18	446	27.4/5.9	28.0/5.7	0.72	0.040	MS/MS	Met	Chlo
12a	Elongation factor 2	XP_003531498.1	643	45	32	1,663	95.0/5.8	81.0/5.7	1.90	0.010	MS/MS	ProtSyn	Cyto
12b	Glutaminyl-tRNA synthetase-like isoform 1	XP_003536678.1	198	15	8	1,473	91.8/5.8	81.0/5.7	1.90	0.010	MS/MS	ProtSyn	Cyto
13	Methionine synthase	NP_001235794.1	820	34	24	1,491	84.4/5.9	70.8/5.7	2.40	0.020	MS/MS	Met	Mito
14	beta-Amylase	BAD93288.1	114	19	6	1,000	56.4/5.3	53.3/6.0	2.72	0.008	MS/MS	Met	Cyto
15	60S ribosomal protein	XP_003526775.1	245	55	10	366	21.8/9.5	23.9/9.8	0.59	0.030	MS/MS	ProtSyn	Cyto
16a	Translation elongation factor 1A-2	ABA12218.1	658	32	17	847	50.0/9.1	61.6/3.9	1.84	0.020	MS/MS	ProtSyn	Cyto
16b	Signal recognition particle 54 kDa protein	XP_002517663.1	146	18	8	717	55.1/9.1	61.6/3.9	1.84	0.020	MS/MS	SigTra	Cyto
17	Actin isoform PEAc14-1	ADP09679.1	462	45	52	754	42.0/5.2	46.9/5.0	1.37	0.030	MS/MS	CellStr	Cyto
18	Proteasome subunit alpha type	XP_002513374.1	396	44	10	453	25.6/5.5	31.0/5.4	0.54	0.004	MS/MS	ProtDest	Cyto
19	Peroxisomal Voltage-dependent anion-selective channel	BAG09368.1	280	51	16	543	29.7/8.5	20.0/8.4	1.98	0.010	MS/MS	Ene	Cyto
20	Glucan endo-1,3-beta-glucosidase	NP_001238474.1	273	38	12	694	38.1/8.7	35.5/8.5	2.07	0.001	MS/MS	DisDef	Chlo
21	Peroxidase	AAD11481.1	116	45	14	360	35.8/8.3	40.0/8.4	2.83	0.020	MS/MS	DisDef	Mito
22	Enolase	AAS18240.1	226	32	8	873	48.0/5.4	51.0/9.5	2.62	0.009	MS/MS	Ene	Cyto

[a] Spot number as given in Fig. 2

[b] Accession number according to the NCBI database

[c] Ions score of identified protein using soybean genome sequence database

[d] Sequence coverage; the proteins with less than 14 % sequence coverage were excluded from the result

[e] The proteins with more than three matched peptides were considered

[f] The score of the high-scoring segment pair from that database sequence

[g] Theo., theoretical; Mr, molecular weight; p*I*, isoelectric point

[h] Exp., experimental

[i] FC, fold change. The protein spots showed a significant change in abundance compared with the control analyzed by LSD test

[j] Indicates the significance of increased and decreased expression of spots according to the *t* test through analysis of variance

[k] The type of MS used in this study. MS means MALDI-TOF MS and MS/MS means nanoLC MS/MS

[l] Function, category using functional classification: ProtDest, protein destination/storage; Met, metabolism; SecMet, secondary metabolism; DisDef, disease/defence; Transcr, transcription; ProtSyn, protein synthesis; SigTra, signal transduction; CellStr, cell structure; Ene, energy and Unclassi, un-classified

[m] Sub-cellular localization, Chlo, chloroplast; Cyto, cytoplasm; Nucl, nucleus and Mito, mitochondria

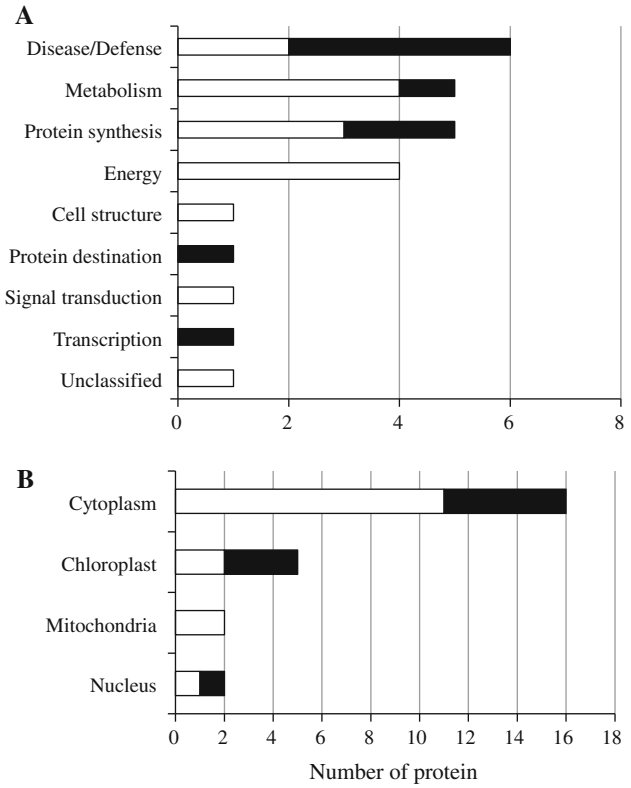

Fig. 3 Classification of differentially changed proteins in soybean roots inoculated with compatible bacteria under flooding stress. The functions of differentially changed proteins were assigned using the classification scheme (Bevan et al. 1998) (**a**). The localization of identified proteins was also classified according to the subcellular localization predicted by WoLF PSORT prediction (Horton et al. 2007) (**b**). *White* and *black columns* represent increased and decreased level of proteins, respectively

proteins, increased under flooding, the increased level of 6 proteins (spots 8, 9, 11, 15, 20 and 22) was suppressed by inoculation. They included phosphoglycerate kinase (spot 8), UDP-glucose 6-dehydrogenase (spot 9), triosephosphate isomerase (spot 11), 60S ribosomal protein (spot 15), glucan endo-1,3-beta-glucosidase (spot 20), and enolase (spot 22) (Table 1).

Three proteins (spots 1, 16 and 21) were increased under flooding and this increase was further enhanced by *B. japonicum* inoculation. They included stress-induced protein SAM22 (spot 1), translation elongation factor 1A-2 and signal recognition particle 54 kDa protein (spot 16), and peroxidase (spot 21).

Analysis of localized abundance of proteins in middle and tip of soybean roots inoculated with compatible bacteria under flooding

In order to confirm the relationship between increase in number and changed morphology of root hairs, and changed proteins in soybean roots inoculated with *B. japonicum* under flooding stress, localized abundance of proteins in middle and tip of soybean roots was analyzed. Root hairs are the site of infection of *B. japonicum* and present abundantly in the middle portion of roots, while root tip is devoid of root hairs. Analysis of localized abundance of six proteins (spots 8, 9, 11, 15, 20 and 22) was carried out in two parts, middle and tip of inoculated soybean roots (Supplementary Fig. 6), without or with subjecting to flooding. Three-day-old soybean was inoculated under flooding. Proteins were extracted from middle and tip of roots at 3 days after treatment and separated by 2-DE (Fig. 5a). Four proteins (spots 8, 9, 20 and 22) and two proteins (spots 11 and 15) were increased and decreased under flooding, respectively. Three proteins (spots 8, 11 and 20) showed differential abundance in middle and tip of soybean roots, whereas three proteins (spots 9, 15 and 22) in middle and tip of roots were changed in response to flooding only (Fig. 5b). These three proteins did not show any change based on their localization in middle and tip of roots.

Discussion

Flooding stress lead to reduced growth and final yield of plants (Sung 1993) and negative impact of flooding have been reported in every aspect of growth in soybean at early growth stage when plants were flooded longer than 1 day (Komatsu et al. 2010). In the present study, the physiological analysis of the soybean seedlings with *B. japonicum* inoculation under flooding stress has revealed that flooding has suppressed the seedling growth of soybean; however, root hair curling was started and their number was increased in inoculated seedlings under flooding. Stage of early symbiotic differentiation is sensitive to flooding stress; however, the interaction of compatible bacteria with plant roots induced the ability to grow under flooding stress in better way than plants without this interaction (Minchin and Summerfield 1976). Little attention has been paid to protein profile of symbiotic interaction under flooding stress specifically during early stage of differentiation. Understanding of the mechanism of plant response during this stage against flooding stress is therefore needed. In present research, proteomics approach was performed to evaluate the effects of flooding stress on early symbiotic interaction between soybean roots and *B. japonicum*.

The effects of flooding stress on soybean at early growth stage are well studied (Komatsu et al. 2009b). The present study was focused to investigate the mechanism of plant response during early symbiotic differentiation under flooding stress. A comparative proteomics analysis in roots of inoculated soybean seedlings with seedlings inoculated under flooding identified 25 proteins. In order to focus this

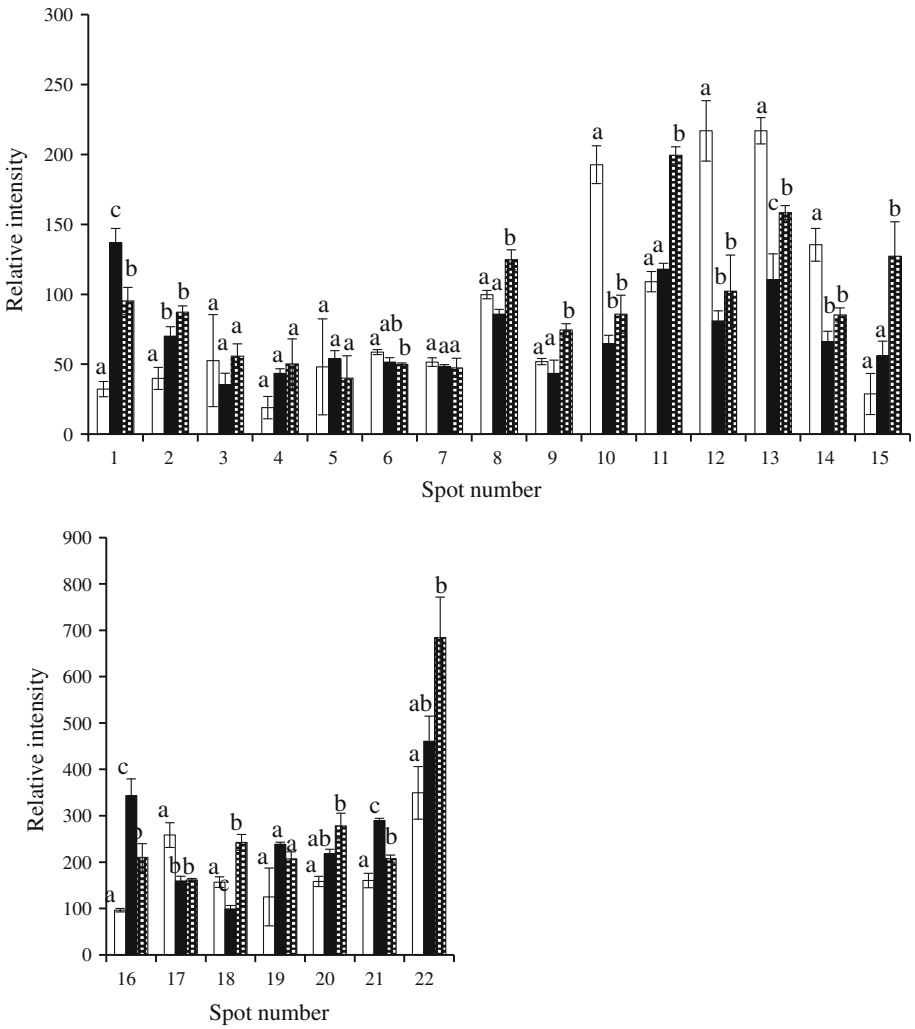

Fig. 4 Comparison of protein abundance in soybean roots among untreated seedlings, inoculated seedlings under flooding, and flooded seedlings. Three-day-old soybean was inoculated with *B. japonicum* followed by flooding. Untreated soybean served as control. After 3 days of treatment, proteins were extracted from roots of untreated seedlings (*white bar*), inoculated seedlings under flooding (*black bar*), and flooded seedlings (*dotted bar*). The differentially changed proteins were quantified using PDQuest software and plotted as the relative intensity. Results are presented as mean ± SE of relative protein intensity for gels from three biological replicates. Statistical analysis was performed by one-way ANOVA Duncan's multiple comparisons test. *Different letters* above the *bars* indicate a statistically significant difference ($P < 0.05$). Protein abundance was analyzed as in Supplementary Fig. 4. Spot numbers are same as in Fig. 2

study to the regulation of response mechanism by *B. japonicum* inoculation in soybean seedlings against flooding, the differential abundance of flood responsive proteins in inoculated seedlings was compared in untreated and flooded seedlings. The flood-responsive proteins, regulated by inoculation, were selected as candidate proteins. These proteins include glucan endo-1, 3-beta-glucosidase, 60S ribosomal protein, phosphoglycerate kinase, UDP-glucose 6-dehydrogenase, triosephosphate isomerase, and enolase. Furthermore, the differential abundance of these proteins was analyzed based on their localization in middle and tip of root.

Glucan endo-1,3-beta-glucosidase was increased in flooded soybean seedlings and regulated by *B. japonicum* inoculation in seedlings under flooding. Mitra and Long (2004) reported the suppression of gene MtBGLU1, which has full-length sequence homology to the glucan endo-1,3-glucosidases during early symbiosis. This suppression was part of a Nod factor signaling pathway downstream of Nod factor recognition because the Nod factor signaling plant mutant dmi1 was defective in the ability to down-regulate MtBGLU1and finally glucan endo-1,3-glucosidase (Mitra and Long 2004). Glucan endo-1,3-beta-glucosidase was induced as part of plant defense; therefore, it can be a marker indicating the initiation of a plant defense response to pathogens (Ward et al. 1991). The suppression of glucan endo-1,3-beta-glucosidase by inoculation may indicate that early symbiotic interaction interferes with plant defense response. 60S ribosomal protein was also increased in flooded soybean seedlings, but this increased level was regulated by inoculation. Protein synthesis is highly energy demanding process and this energy permits high fidelity in the biological translation of mRNA into the amino acid sequence of proteins (Nelson and Cox 2004a). Bailey-Serres and Freeling (1990) reported that dephosphorylation of ribosomal proteins in maize roots under oxygen deprivation condition may lead to the reduction in binding sites of ribosome, required for protein synthesis (Bailey-Serres and Freeling 1990). Results from the present study indicate that to fulfill the required number of binding sites on

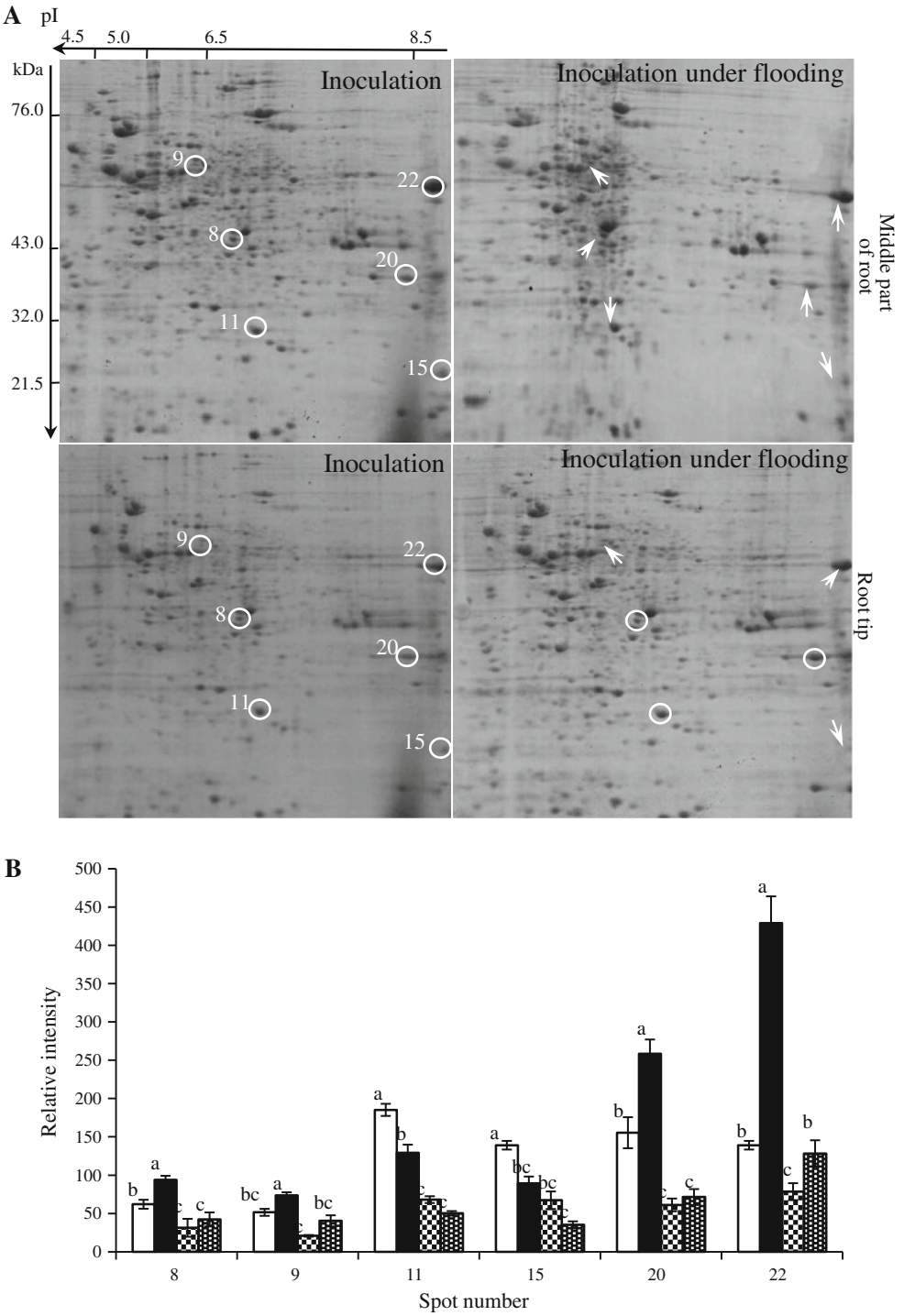

Fig. 5 2-DE pattern and abundance level of candidate proteins in middle portion and tip of soybean roots inoculated with compatible bacteria subjected or not subjected to flooding. Three-day-old soybean was inoculated with *B. japonicum* followed by flooding. Proteins were extracted from middle portions of inoculated roots without (*white bar*) or with (*black bars*) flooding and tips of inoculated roots without (*squared*) or with (*dotted bars*) flooding, separated by 2-DE and stained by CBB (**a**). Six protein spots were quantified using PDQuest software and plotted as the relative intensity (**b**). Statistical analysis was performed by one-way ANOVA Duncan's multiple comparisons test. *Different letters* above the *bars* indicate a statistically significant difference ($P < 0.05$). Spot numbers are same as in Fig. 2

ribosomes, the number of 60S ribosome was increased in flooded seedlings. However, the chemical signals by inoculation (Lerouge et al. 1990) may affect the ribosomal structure, which restored the number of binding sites and thus regulated the 60S ribosome level.

Phosphoglycerate kinase is a well-known ATP-generating enzyme that is a part of glycolytic pathway (Banks et al. 1979). In addition to glycolysis, phosphoglycerate kinase involved in regulation of cellular processes including primer recognition during DNA synthesis (Jindal and Vishwanatha 1990) and mRNA transcription at the elongation step, probably through the interaction with tubulin (Ogino et al. 1999). In the present study, phosphoglycerate kinase was increased under flooding but this increased level was regulated by *B. japonicum* inoculation in seedlings under flooding. The results from this study suggest

that phosphoglycerate kinase might be involved in DNA synthesis and mRNA transcription, which may lead to high cellular activity during early symbiotic differentiation. Increase in number of root hair as a result of infection by *B. japonicum* on soybean roots supports this idea.

UDP-glucose dehydrogenase, which is a metabolism-related protein, was increased under flooding stress (Nanjo et al. 2010). UDP-glucose dehydrogenase catalyzes the conversion of UDP-glucose to UGP-glucuronate that is potentially involved in synthesis of hemicelluloses (Tenhaken and Thulke 1996). Recently, Muszynski et al. (2011) has reported the involvement of UDP-glucose dehydrogenase in synthesis of rhizobial lipopolysaccharide that is required to establish an effective symbiosis with its host plant. In the present study, the increased level of UDP-glucose dehydrogenase was suppressed by inoculation in flooded seedlings. This suppression indicates the utilization of UDP-glucose dehydrogenase in lipopolysaccharide synthesis to facilitate the signaling during early symbiotic interaction between *B. japonicum* and soybean roots.

Triose-phosphate isomerase and enolase are important enzymes of glycolysis (Nelson and Cox 2004b). Triose-phosphate isomerase catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, whereas enolase is the key enzyme of energy generating step of glycolysis which converts 2-phosphoglycerate into phosphoenol-pyruvate (Nelson and Cox 2004b). Chen and Thelen (2010) have reported the role of triose-phosphate isomerase in the integration of seed reserve mobilization and seedling establishment. Increased level of glycolysis-related proteins help plants to cope with energy crises imposed by flooding stress (Hashiguchi et al. 2009). In the present study, triose-phosphate isomerase and enolase were increased under flooding stress; however, this increase was regulated by inoculation with *B. japonicum.* This regulation of consumption of energy resources may be a better strategy for economic and efficient uses of cell resources, that produce energy only when it is needed depending on physiological state of plants.

This study represents the first evidence of beneficial effects of inoculation with *B. japonicum* on soybean against flooding injury. These results indicate that six proteins, increased under flooding, were regulated equal or near to untreated seedlings by inoculation. These proteins include glucan endo-1,3-beta-glucosidase, 60S ribosomal protein, phosphoglycerate kinase, UDP-glucose 6-dehydrogenase, triosephosphate isomerase, and enolase related to disease/defense, protein synthesis, energy, and metabolism. The results from physiological and proteomics analyses indicate that inoculation modulates the disease/defense, energy, and metabolism-related proteins, which lead to root hair curling and increase in their number. Regulation of energy and disease/defense-related proteins during symbiotic differentiation had been described previously (Jindal and Vishwanatha 1990; Mitra and Long 2004). The role of three key proteins, which are glucan endo-1,3-beta-glucosidase, phosphoglycerate kinase, and triosephosphate isomerase, in the regulation of various developmental processes had also been reported (Zhao and Assmann 2011; Chen and Thelen 2010). In the present study, regulation of these enzymes by inoculation in flooded seedlings suggested that level of these enzymes, as in untreated seedlings, is needed to initiate the symbiotic interaction in soybean seedlings when inoculated with *B. japonicum*.

This study was conducted to investigate whether the inoculation with *B. japonicum* has alleviating effects from flooding injuries during early symbiotic differentiation. The results from the present study indicated that glucan endo-1,3-beta-glucosidase, phosphoglycerate kinase, and triosephosphate isomerase were regulated by inoculation in flooded soybean seedlings, which suggest that although flooding stress suppressed the root hair number and growth required for the early symbiotic events in soybean roots, inoculation can alleviate this suppression partially by regulating the enzymes during symbiotic differentiation. However, symbiotic differentiation was not started in untreated soybean seedlings because some chemical signals released by plants and bacteria are required to start this process (Lerouge et al. 1990). The differential abundance of glucan endo-1,3-beta-glucosidase, phosphoglycerate kinase, and triosephosphate isomerase in middle and tip of root further indicates the relationship of these proteins with root hair number and their changed morphology.

These results suggest that disease/defense, energy, and metabolism-related proteins may be particularly subjected to regulation in flooded soybean seedlings, when inoculated with *B. japonicum.* This regulation might help plants to perform better under flooding stress. Proteomics technique revealed the changes in abundance pattern of proteins including various enzymes, co-enzymes, and molecular chaperons, which regulate various molecular processes, thus resulting in physiological and morphological changes. Although proteomics is capable to unravel the molecular basis of physiological changes, however, these results need more confirmatory experiments using mutants deficient of the genes encoding these proteins and over expressed transgenic soybeans for further verification.

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Conflict of interest The authors declare that they have no conflict of interest.

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